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Nickel ion concentrations in the saliva of patients treated with self-ligating fixed appliances: a prospective cohort study

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Abstract: OBJECTIVE Orthodontic appliances are considered to be highly biocompatible although adverse effects attributed to the release of nickel ions (Ni(2+)) have been documented. Self-ligating brackets have grown in popularity for economic reasons and supposed friction reduction. The aim of the present prospective cohort study was therefore to determine salivary Ni(2+) concentrations in patients undergoing orthodontic treatment with self-ligating fixed appliances. MATERIALS AND METHODS A group of 30 patients between 10 and 13 years of age were treated with self-ligating brackets (SmartClip™), molar bands, and nickel-titanium (NiTi) archwires. Unstimulated saliva samples were collected after different time points (before treatment, after self-ligating bracket and band placement, before archwire insertion, after archwire insertion, and finally 4 and 8 weeks afterwards) and analyzed with an ICP mass spectrometer followed by generalized estimating equation modelling with = 5 %. RESULTS The baseline median salivary Ni(2+) concentration was 21.85 µg/l, while the Ni(2+) concentrations at the following visits ranged between 13.73 and 85.34 µg/l. Significant increases in Ni(2+) levels compared to the baseline levels were detected after band/bracket placement [+59.76 µg/l; 95 % confidence interval (CI) 44.88-74.64 µg/l; P < 0.001] and after archwire insertion (+53.55 µg/l; 95 % CI 25.57-81.52 µg/l; P < 0.001). After 4 weeks, Ni(2+) concentrations returned to initial control levels or were lower. CONCLUSION Self-ligating orthodontic appliances may affect salivary Ni(2+) concentrations in vivo over the short term. However, levels resembled those documented in conjunction with conventional bracket use and remained below the daily dietary Ni intake.

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Title Page

Nickel ion concentrations in saliva of patients treated with self-ligating fixed appliances: a prospective cohort study

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Short title: *In-vivo* prospective study of nickel release using self-ligating multibracket appliances.

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Abstract

Background and objective: Orthodontic appliances are considered to be highly biocompatible, even though adverse effects due to the release of nickel ions (Ni^{+2}) have been documented. Self-ligating brackets have grown in popularity due to economic aspects and reputed friction reduction. Aim of the present study was therefore, to determine the salivary Ni^{+2} concentrations in patients undergoing orthodontic treatment with self-ligating fixed appliances.

Material and Methods: A group of 30 patients (10-13 years) were treated with self-ligating brackets (SmartClip™, 3M Unitec, 0,022 inch slot), stainless-steel molar bands (Ormco) and nickel-titanium archwires (Smile Dental, 0,014 inch). Unstimulated saliva samples were collected after different time-points (before treatment, after self-ligating bracket and band placement, before archwire insertion, after archwire insertion, and finally 4 and 8 weeks afterwards) and analysed with an ICP mass spectrometer followed by non-parametric statistics at 5%.

Results: The baseline median salivary Ni^{+2} concentration was 21.85 µg/l, while the Ni^{+2} concentrations for the following visits ranged between 13.73 µg/l and 85.34 µg/l. Significant increases of Ni^{+2} levels compared to the baseline levels were detected after band/bracket placement (+59.76 µg/l; 95% confidence interval: 44.88 to 74.64 µg/l; $P<0.001$) and after archwire insertion (+53.55 µg/l; 95% confidence interval: 25.57 to 81.52 µg/l; $P<0.001$). After 4 weeks, Ni^{+2} concentrations returned to initial control levels.

Conclusions: To conclude, self-ligating orthodontic appliances may affect the salivary Ni^{+2} concentrations *in-vivo*. However, levels were similar to conventional brackets and remained below the daily dietary Ni intake.

Words: 238

Keywords: nickel release, corrosion, self-ligating fixed appliance, saliva, ICP mass spectrometry, biocompatibility

Zusammenfassung

Hintergrund und Ziel: Kieferorthopädische Apparaturen werden allgemein als hoch biokompatibel eingeschätzt, obwohl verschiedene Nebenwirkungen bedingt durch die Freisetzung von Nickelionen (Ni^{+2}) dokumentiert wurden. Selbstligierende Bracketsysteme erfreuen sich zunehmender Beliebtheit aufgrund wirtschaftlicher Aspekte und propagierter Friktionsreduktion. Ziel der vorliegenden prospektiven Kohortenstudie was es daher, die Ni^{+2} Konzentrationen im Speichel von Patienten zu bestimmen, die einer Behandlung mit selbstliegenden festsitzenden Multibracketapparaturen unterzogen wurden.

Material und Methodik: 30 Patienten (10-13 Jahre) wurden mit selbstligierenden Brackets (SmartClip™, 3M Unitec, 0,022 inch slot), Molarenbändern (stainless-steel, Ormco) und Nickel-titanium Bögen (Smile Dental, 0,014 inch) behandelt. Unstimulierte Speichelproben wurden nach unterschiedlichen Zeitpunkten (vor Behandlung, nach Insertion selbstligierender Brackets/Bänder, 2 Wochen nach und direkt vor Bogeninsertion, direkt nach Bogeninsertion, 4 und 8 Wochen nach Bogeninsertion) gesammelt und mittels ICP Massenspektrometer analysiert. Die Daten wurden non-parametrisch mit einer Signifikanzgrenze von 5% ausgewertet.

Ergebnisse: Der Medianwert der Nickelkonzentrationen im Speichel vor der Behandlung (Referenzwert) lag bei 21.85 µg/l. In den folgenden Sitzungen lagen die Ni^{+2} Werte zwischen 13.73 µg/l und 85.34 µg/l. Ein signifikanter Anstieg der Ni^{+2} Konzentrationen im Vergleich zum Referenzwert wurde nach der Insertion der selbstligierenden Brackets und Bänder (+59.76 µg/l 95% Konfidenzintervall: 44.88 to 74.64 µg/l; $P<0.001$) und nach Bogeninsertion (+53.55 µg/l; 95% Konfidenzintervall: 25.57 to 81.52 µg/l; $P<0.001$) detektiert. Nach vier Wochen waren die Ni^{+2} Werte wieder auf dem Referenzniveau.

Schlussfolgerungen: Die Daten lassen die Schlussfolgerungen zu, dass selbstligierenden Multibracketsysteme die Nickelkonzentration im Speichel *in-vivo* beeinträchtigen. Dennoch lagen die Werte in Höhe derer konventioneller Multibracketsysteme sowie unterhalb der täglichen Nickelaufnahme durch die Nahrung.

Worte: 247

Schlüsselworte: Nickelfreisetzung, Korrosion, Selbstligierendes Multibracketsystem, Speichel, ICP Massenspektrometrie, Biokompatibilität

Main text

Nickel ion concentrations in saliva of patients treated with self-ligating fixed appliances: a prospective cohort study

Introduction

Nickel (Ni) is one of the main components in contemporary orthodontic appliances [4]. Its content ranges from 8% up to more than 50% in stainless steel and nickel titanium (NiTi) alloys, respectively. Although most orthodontic materials are considered to be highly biocompatible, *in-vitro* and *in-vivo* studies indicate that a certain degree of intra-oral corrosion is inevitable leading to the release of Ni ions (Ni^{+2}) [1, 13, 16, 23-25, 27]. Thereby, various factors such as temperature variations, mechanical stress induced by chewing or grinding, pH changes, bacterial accumulation, psychological stress and to a lesser extent the actual Ni content of orthodontic materials [3, 6, 21, 24] seem to determine the degree of Ni^{+2} release.

In the last years self-ligating brackets have quickly permeated the orthodontic market, partly due to successful marketing and secondarily due to claims of time reduction and clinical superiority, even though evidence is lacking [8]. These brackets are characterized by an increase of volume in comparison with conventional brackets and an irregular morphology due to the clip-connection system. Contemporary self-ligating brackets can be divided into an active or passive nickel-containing clip that holds the archwire engaged into the bracket slot. Apart from the possible additional release of Ni^{+2} from the clip, this clip also enables an active and constant surface proximity between wire and bracket components which may result in increasing Ni^{+2} levels *in-vivo*. However, to date only one study has investigated the release of self-ligating fixed appliances *in-vivo* using ten patients in the group with self-ligating brackets [40].

As a consequence of metal leach orthodontic appliances are under suspicion to induce various side effects. Case reports of allergic reactions during orthodontic therapy support this suspect [11, 26]. Moreover, *in-vitro* evidence indicates that Ni can cause cytotoxic, immunogenic, mutagenic or carcinogenic processes in dependence on the chemical form, concentration, duration and route of exposure [9, 14, 18, 30, 47]. The most common adverse effect in orthodontics is the induction of allergic reactions and Ni is by far the most common contact allergen [45]. The prevalence of Ni hypersensitivity in industrial countries seems to have increased steadily differing between male (3-8%) and female patients (more than 30%) [24, 39, 45]. Even though Ni allergy is frequently observed during skin contact, allergic reactions in the oral cavity are rare [7, 43]. In addition, there is evidence of a reduced prevalence of Ni hypersensitivity in patients having received orthodontic treatment before ear piercing [15, 19]. Interestingly, new insights in pathomechanism of Ni hypersensitivity have revealed that a specific innate immune receptor (Toll-like receptor 4) is implicated in this event [42]. Toll-like receptors are highly conserved and ubiquitously expressed receptors, which sense microbial pathogens as well as endogenous ligands playing a pivotal role in host immune defence. Anyhow, orthodontic materials containing Ni may cause allergic reactions in Ni-sensitive patients and the use of self-ligating fixed appliances may increase Ni^{+2} levels *in-vivo* reinforcing the demand of investigations in this field.

The primary aim of this study was therefore, to determine the amount of Ni^{+2} in the saliva of patients treated with fixed orthodontic appliances comprising commonly used self-ligating brackets (SmartClip™ made of stainless steel with two passive nickel titianium (NiTi) clips), molar bands made of stainless steel and NiTi archwires. Results were compared with those of Petoumenou *et al.* using the same experimental setting with a conventional fixed system [37]. We hypothesized that self-ligating fixed appliances induce higher Ni^{+2} levels compared to conventional appliances due to the larger volume in comparison with conventional brackets and the clip mechanism.

Materials and Method

Patient and sample management

Eligible patients for this study were selected from the Department of Orthodontics, University of Bonn, according to the following inclusion criteria: (i) no age restriction, (ii) no previous orthodontic treatment, and (iii) no previous allergic reaction to Ni. Excluded were: (i) patients with metal restorations, (ii) patients with systemic diseases or (iii) smokers. A group of thirty patients fulfilled the inclusion criteria (10-13 years)

Orthodontic treatment protocol

This study was designed as a pragmatic trial, as no study-specific protocol was used for the treatment planning or conduct.

A self-ligating fixed appliance was administered to all patients following the standard protocol of the Department of Orthodontics, University of Bonn (Supplement 1). A self-ligating bracket system (made of stainless steel with a passive NiTi clip 0.56 mm (0.022-inch), SmartClip™, 3M Unitek, Monrovia, CA) was used for all patients with fitted stainless steel bands on all first molars (18-21 brackets and 4 bands per patient). After placement of the fixed appliance, a 0.36 mm (0.014-inch) martensitic-active NiTi archwire (True Form, smile dental, Düsseldorf, Germany) was fully engaged at all teeth of the upper and the lower dentition. Wire stops were used in all cases to avoid sliding of the archwire, while special mechanics were used in some patients (including springs for space management or bite planes) and documented in detail. All fixed appliance materials together with their element composition are listed in Table 1.

Sample acquisition and analysis

The protocol for the collection of saliva samples from the patients was based on previous studies [37, 38]. Patients were instructed to avoid rinsing and eating at least 30 minutes before sampling. Unstimulated saliva samples were collected from each patient in sterile 50 ml propylene tubes (Cellstar^{TM}; Greiner Bio-One, Frickenhausen, Germany) by an un-blinded clinician (ACK) at six consecutive time-points: T1: before orthodontic treatment, T2: directly after placement of the self-ligating fixed appliance (i.e. self-ligating brackets/band placement without archwire), T3: 2 weeks later and before archwire insertion, T4: directly after archwire insertion, T5: 4 weeks after archwire insertion, and T6: 8 weeks after archwire insertion (Fig. 1).

Samples were then transferred to glass tubes (VWR International GmbH, Darmstadt, Germany) and stored at –20ºC until. From every sample, 1 ml of saliva was put into an open glass vessel and dried using an infrared lamp (InfraPhil HP3616; Philips, Eindhoven, The Netherlands) for almost 12 hours. For organic matter digestion, 0.2 ml of aqua regia (3 parts hydrochloric acid to 1 part nitric acid) was added; the vessels were closed and left for 24 hours. Afterwards, the solution was diluted to a volume of 3 ml (Ampuwa water, Fresenius Kabi, Bad Homburg, Germany) and filtered (FP30, 1.2CA, Schleicher & Schuell, Dassel, Germany). Ni^{+2} levels were analysed using an inductively-coupled plasma mass spectrometer (SCIEX ELAN 5000; Perkin Elmer, Wellesley, MA, USA). Each sample was examined consecutively three times and the mean of the three measurements was taken. Results were converted according to the calibration standard (`Standard water 1`10090a307; Merck KGaA, Darmstadt, Germany). The detection limit of the machine was 0.1 µg/l or 1 atomic mass unit [44]. Regular calibration of the instrument reinsured the detection limit and high resolution of the apparatus. Reference measurements taken with Ampuwa water were subtracted from the results.

Statistical analysis

Normality of the data was tested through histograms' inspection and with the Kolmogorov-Smirnov test with Lilliefors correction. As data were not normally-distributed and inhomogeneous, descriptive statistics included the median, range and the interquartile range (Q1-Q3). Mann-Whitney tests were conducted to compare the Ni^{+2} concentrations between time-points. Multivariate mixed-effects Generalised Estimating Equation (GEE) models (Poisson family) were fitted to compare the Ni^{+2} levels at the various time-points as clustering variables. Model fit was assessed with the quasi-likelihood independence model criterion statistic proposed by Pan [34]. All analyses were performed in Stata version 10 (StataCorp LP, College Station, TX) with the macros *randsum* and *xtgee*. All *P* values were 2-sided with a level of significance at $\alpha = 0.05$

Results

Salivary Ni^{+2} concentrations

Salivary Ni^{+2} levels measured at all time-points are demonstrated in Table 2 and Fig. 2. The baseline median levels of Ni^{+2} (before placement of the self-ligating fixed appliance) were 21.85 μg/l (reference) (Table 2 and Fig. 3). Median salivary Ni^{+2} concentrations ranged between 13.73 and 85.34 μg/l in accordance with time-points (Table 2). In detail, compared to baseline levels (21.85 μg/l) Ni^{+2} levels were elevated after placement of the self-ligating appliance (85.34 μg/l), returned to baseline levels after two weeks (19.19 μg/l), were again elevated after placement of the NiTi archwires (57.74 μg/l) and returned again to baseline levels or lower after 4 weeks (13.73 μg/l).

The results of the GEE modelling using patient and time-point as the clustering variables is seen in Table 3. In the fitted model, only the factor "time-point" influenced significantly the Ni^{+2} concentrations and the predicted Ni^{+2} levels are seen in Fig. 4. The *post hoc* comparisons among the various time-points (Table 4) indicated that significantly elevated Ni^{+2} levels compared to the baseline levels (T1) were found after placement of the self-ligating fixed appliance (T2; difference: 59.76 μg/l; 95% confidence interval: 44.88 to 74.64 μg/l; $P < 0.001$) and after the insertion of the NiTi archwires (T4; difference: 53.55 μg/l; 95% confidence interval: 25.57 to 81.52 μg/l; $P < 0.001$). On the contrary, 4 weeks after insertion of the NiTi archwires (T5) the Ni^{+2} levels were significantly reduced compared to the baseline levels (T1) (difference: -7.75 μg/l; 95% confidence interval: -13.86, -1.63 μg/l; $P = 0.013$)

Discussion

Investigations about adverse effects of orthodontic treatment have been widely conducted in the last years, reinforced by possible implications of Ni-containing orthodontic appliances [18], the increasing prevalence of Ni allergy [20, 39, 45] and indications of intraoral aging of orthodontic materials [12, 33]. Active and passive self-ligating devices were introduced the last decade to presumably improve sliding mechanics by the reduction of frictional forces. This was linked to enhanced tooth movement and reduced treatment duration. Even though, the scientific evidence for the benefit is still lacking, several orthodontists use these appliances possibly due to economic aspects [8]. In the present study we determined salivary Ni concentrations in patients treated with self-ligating fixed appliances (commonly used self-ligating brackets made of stainless steel and two passive NiTi clips, stainless steel bands as well as NiTi archwires) with Ni content ranging between 5% and 55% (Table 1). A well-established analysis method with high detection limits concerning Ni^{+2} levels in fluids was used in accordance with previous investigations [5, 37, 38].

The results of the current study are in line with Sahoo *et al.* who evaluated salivary Ni^{+2} and chromium concentrations in twenty women divided in two groups [40]. In group 1, only ten subjects were treated with conventional (MBT preadjusted edgewise brackets) fixed appliance system and additional ten patients received self-ligating (SmartClip™) fixed appliance system (group 2). Both groups had 16 brackets, four bands as well as 0.016 inch NiTi wires (3M Unitec) and unstimulated saliva samples were collected 1 hour before, 1, 7 and 30 days after placement of appliances [40]. In contrast to our investigation, the authors used an atomic absorption spectrometer to determine Ni^{+2} levels in patients` saliva [40]. Atomic absorption spectrometer has a higher detection threshold and inferior resolution than inductively-coupled plasma mass spectrometry [5, 27, 37, 38, 44]. Therefore, differences in methodology, sample size and time-points for sample collection might explain their generally reduced Ni^{+2} concentrations varying between 0.689 to 2.895 µg/l in group 1, and 0.680 to 4.950 µg/l in group 2 with self-ligating brackets. However, they detected the same percentage of Ni^{+2} increase after the insertion of self-ligating fixed appliances as found in the present study. The higher Ni^{+2} levels in the self-ligating group compared to the conventional group 1 were attributed to the NiTi clips which could be an additional source of nickel [40].

In terms of differences between self-ligating and conventional fixed appliances, direct comparison can be made with the study of Petoumenou *et al*. [37]. They used a similar protocol to assess Ni^{+2} levels in the saliva of 18 patients treated with conventional stainless steel brackets and bands (Ormco) as well as 0.016 inch NiTi archwires in both jaws. They reported baseline median Ni^{+2} concentrations of 34 µg/l (before appliance placement), which were significantly increased after placement of the fixed appliance (78 µg/l) and after the insertion of the archwires (56 µg/l) [37]. The results of the present were consistent with the study of Petoumenou *et al.* [37], although a higher dispersion of data was measured, possibly due to sample or appliance differences. Large variations in the measured Ni^{+2} levels of orthodontic patients were also reported by Sahoo *et al.* [40] and Matos de Souza *et al*. [29], who evaluated 30 patients with orthodontic appliances using inductively-coupled plasma mass spectrometry. This variation was attributed to the effect of dietary intake of Ni^{+2} or measurements at different time-points within a day [29]. Mechanical stress induced by chewing or grinding, changes of the pH, microbial oral flora and psychological stress might also influence the intraoral Ni^{+2} release [3, 6, 21, 24, 35]. Finally, frictional forces between the arch and the clips as well as the larger volume of self-ligating brackets might favour intraoral metal leaching.

In-vitro analysis of several self-ligating brackets revealed Ni^{+2} levels between 0.01 and 5.24 µg/day in a single measurement, thereby SmartClip™ brackets belonged to the group with the lowest Ni^{+2} levels [27]. Their data were confirmed by a current *in-vitro* study [28]. However, they detected increased nickel levels in the self-ligating group with SmartClips™ compared to the conventional brackets from the same manufacturer (0.11 ppm versus 0.00 ppm) [28]. Surprisingly, SmartClips™ displayed the lowest nickel release after the respective conventional bracket group, even lower as the other conventional bracket systems from distinct manufacturers. Anyhow, the authors concluded that the aging process was not differing between groups [28] and these studies were performed without archwires which does not represent the clinical situation. Moreover, the absence of frictional forces between brackets and archwires may lead to reduced Ni^{+2} levels.

Concerning the duration of exposure, some studies indicate that metal leaching processes might be time-dependent [6, 36]. High metal ion levels are found one to two weeks after exposure to metal appliances and then revert to the initial levels [1, 6, 23, 37], thereby hypothesising the formation of a passivation layer that reduces the release of ions. This initial increase of Ni^{+2} levels followed by normalisation after two weeks was also seen in the present study and in the investigation of Sahoo *et al.* (Fig. 2) [40].

Finally, adverse effects of orthodontic appliances might also include cytotoxic, inflammatory or allergic processes due to the release of other metal ions like cobalt, chromium or copper [2, 3, 22, 24, 41, 45]. Hence, side effects associated with orthodontic appliances might not always be the result of Ni-induced mechanisms via Toll-like receptor 4 activation. However, Ni is the main sensitizer for contact hypersensitivity and, therefore, often blamed for adverse reactions during orthodontic therapy. On the other side, a current meta-analysis indicated that orthodontic treatment may even reduce the incidence of Ni hypersensitivity, when the treatment preceded Ni sensitization through ear piercings [19]. Cases of Ni-induced allergic reaction during orthodontic therapy are rare and often related to extraoral appliances, as dermal Ni contact seems to be able to induce hypersensitivity more easily than the contact with oral mucosa. This might be associated with the functional specificity of dendritic cells, which are the main antigen-presenting cells and play a crucial role in the initial phase of contact hypersensitivity induction [32]. Novak *et al.* showed that oral dendritic cells possess a more tolerogenic character, whereas dermal dendritic cells are primarily characterised by their pro-allergenic properties supporting the development of Ni allergy [32]. Small and frequent oral Ni^{+2} concentrations during orthodontic treatment before Ni sensitization may therefore favour the induction of an oral tolerance. In the present investigation detected Ni^{+2} levels were below the daily oral dietary intake of 200 to 600 µg, which is generally accepted and in line with previous studies analysing conventional fixed appliance system [10, 31, 46].

Strengths and limitations

The strengths of this study include (i) its *in-vivo* nature, which reflects better the clinical reality compared to *in-vitro* studies of ion leaching from self-ligating fixed appliances [12], (ii) the use of a standardized protocol for the collection and analysis of the saliva samples, and (iii) the clinical use of self-ligating brackets for fixed orthodontic treatment as its popularity has increased in practice over the last decade. As no specific inclusion criteria were used during patient recruitment, the results of this study can probably be generalised to the average orthodontic patient treated with metallic self-ligating fixed appliances. The limitations of this study include (a) possible confounding in the measurement of Ni^{+2} from dietary or other sources and (b) the fact that Ni^{+2} levels are not directly linked to biologic adverse reactions to nickel.

Conclusions

Median salivary Ni^{+2} concentrations in patients with self-ligating fixed appliances ranged between 13.73 and 85.34 μg/l, remaining below the daily dietary nickel intake. The highest levels of Ni^{+2} were measured after the placement of the self-ligating brackets and bands as well as NiTi archwires insertion which returned to baseline levels after a period of two weeks. Finally, we detected similar Ni^{+2} levels documented for conventional fixed appliances discounting our hypothesis. The small amounts of nickel ions released by orthodontic materials might support the idea of an orally-induced tolerance against nickel during early orthodontic treatment.

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TABLES

Table 1. Composition of the materials used in the study (according to manufacturer).

		Content of metal elements in per cent (%)															
Material	Product	Ni	C	Si	Mn	Cr	P	S	Fe	Mo	W	Ti	Co	Cu	NB+Ta	O	H
Brackets	SmartClip; 3M Unitek, Monrovia, CA	5.00-13.00	< 0.07	< 1.00	< 1.00	17.00	< 0.04	< 0.03	72.00	-	-	-	-	4.00	0.30	-	-
Bands	Ormco, Glendora, CA	10.50-13.00	0.12	1.00	2.00%	17.00-19.00	0.05	0.03	rest	-	-	-	-	-	-	-	-
Martensitic-active NiTi archwires	Smile Dental, Düsseldorf, Germany	55.00	≤ 0.50	-	-	-	-	-	-	-	-	rest	-	-	-	≤ 0.50	≤ 0.50
NiTi open spring	Ormco, Glendora, CA	54.00-56.00	-	-	-	-	-	-	-	-	-	rest	-	-	-	-	-
SS closed coil spring	Ormco, Glendora, CA	8.00-10.00	0.15	1.00	2.00	17.00-19.00	0.05	0.03	rest	-	-	-	-	-	-	-	-

Table 2. Ni^{2+} concentrations (µg/l) according to each time-point.

Time-point (patients)	Median	Interquartile range	Range
T1 (n=30)	21.85	15.95-27.79	12.05-56.65
T2 (n=30)	85.34	52.74-113.17	18.82-172.13
T3 (n=30)	19.19	17.81-25.72	11.24-143.53
T4 (n=30)	57.74	25.88-91.99	7.48-343.65
T5 (n=29)	13.73	10.50-20.59	4.70-49.60
T6 (n=30)	19.83	16.92-24.12	8.17-42.17

Table 3. Results of the generalized estimating equation modelling.*

Factor	Coefficient	Standard error	P-value
Time-point	-7.45	0.76	< 0.001
Constant	69.82	5.29	< 0.001

*Model information: clustering variables: patient and time-point; link: identity; family: Poisson; correlation: autoregressive; Wald $chi^2 = 96.70$; *P*-value < 0.001; Trace = 55.106; Quasi-likelihood independence model criterion = -41762.336.

Table 4. *Post hoc* comparisons of Ni^{2+} concentrations (μg/l) among the various time-points.

Time-point	**Difference (95% CI)**	***P*-value**
T1	*Reference*	
T2	59.76 (44.88, 74.64)	< 0.001
T3	0.70 (-8.65, 10.05)	0.883
T4	53.55 (25.57, 81.52)	< 0.001
T5	-7.75 (-13.86,-1.63)	0.013
T6	-4.30 (-10.03, 1.43)	0.142

CI, confidence interval.

Figure legends

Fig. 1. Sample management according to time-points (T)

Fig. 1. Probenmanagement in Abhängigkeit vom Zeitpunkt der Probenentnahme (T)

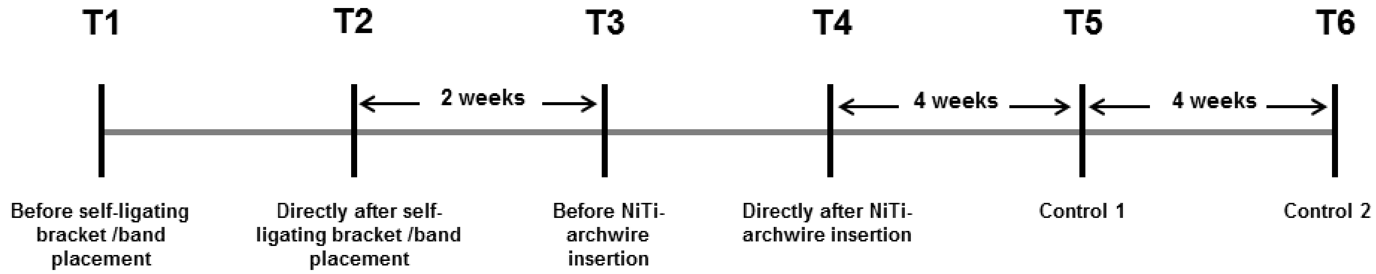

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Fig. 2. Line plot of nickel concentration in the saliva of all patients according to sample collection: T1, reference standard; T2, after self-ligating bracket/band placement; T3, 2 weeks later and before wire insertion; T4, directly after wire insertion; T5, 4 weeks after wire insertion; T6, 8 weeks after wire insertion.

Fig. 2. Liniendiagram zur Darstellung der Nickelkonzentrationen im Speichel aller Patienten in Abhängigkeit vom Zeitpunkt der Probenentnahme: T1, Referenzstandard; T2, nach Insertion selbstligierender Brackets/Bänder; T3, 2 Wochen nach und direkt vor Bogeninsertion; T4, direkt nach Bogeninsertion; T5, 4 Wochen nach Bogeninsertion; T6, 8 Wochen nach Bogeninsertion.

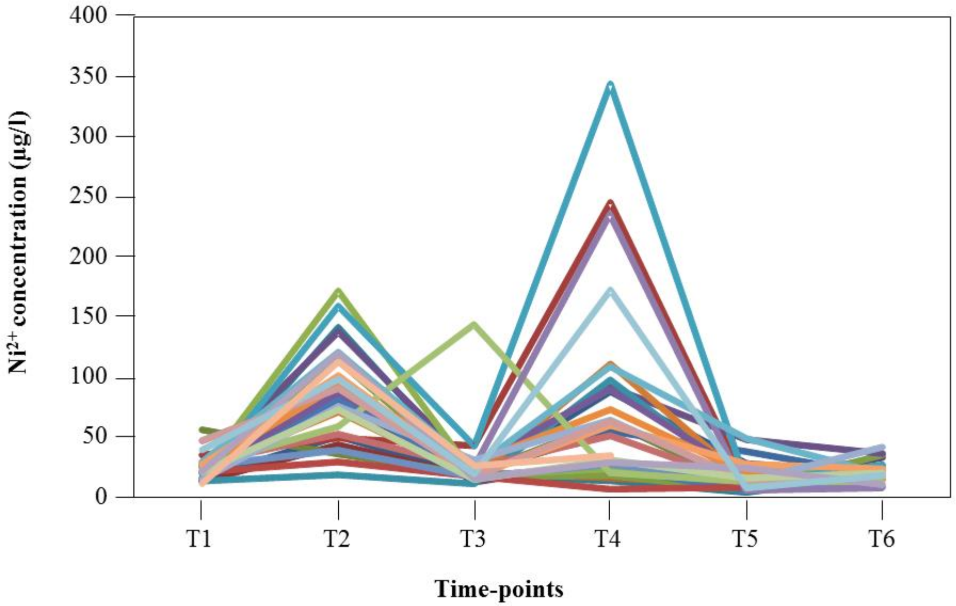

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Fig. 3. Box-and-whisker diagram of nickel concentration in the saliva of all patients (n = 30) according to time of sample collection: T1, reference standard; T2, after self-ligating bracket/band placement; T3, 2 weeks later and before wire insertion; T4, directly after wire insertion; T5, 4 weeks after wire insertion; T6, 8 weeks after wire insertion.

Fig. 3. Boxplot zur Darstellung der Nickelkonzentrationen im Speichel aller Patienten (n =30) in Abhängigkeit vom Zeitpunkt der Probenentnahme: T1, Referenzstandard; T2, nach Insertion selbstligierender Brackets/Bänder; T3, 2 Wochen nach und direkt vor Bogeninsertion; T4, direkt nach Bogeninsertion; T5, 4 Wochen nach Bogeninsertion; T6, 8 Wochen nach Bogeninsertion.

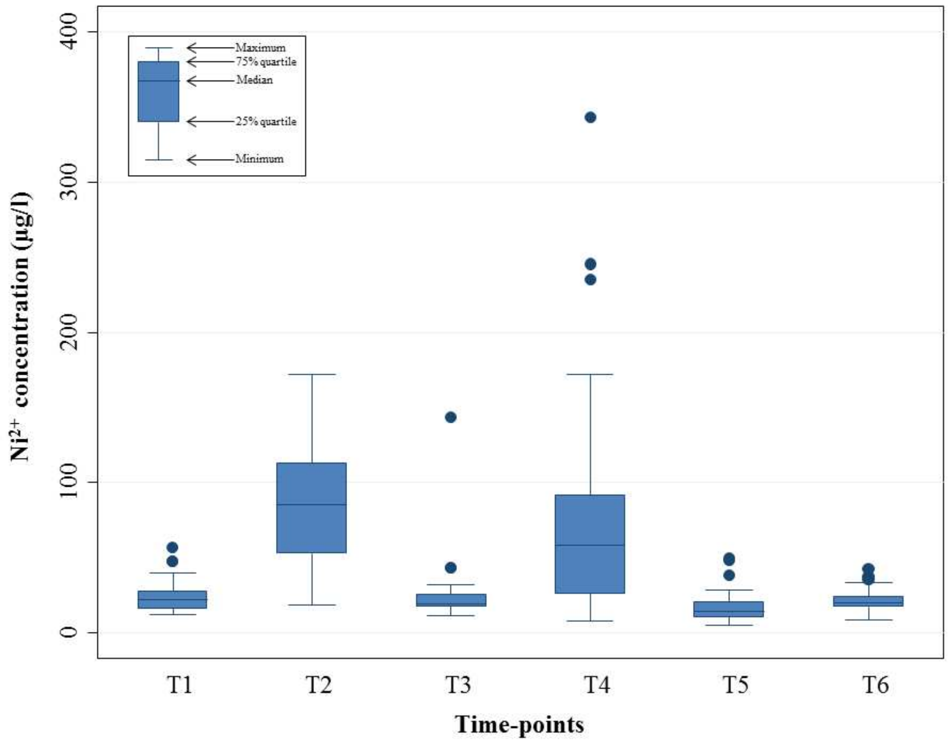

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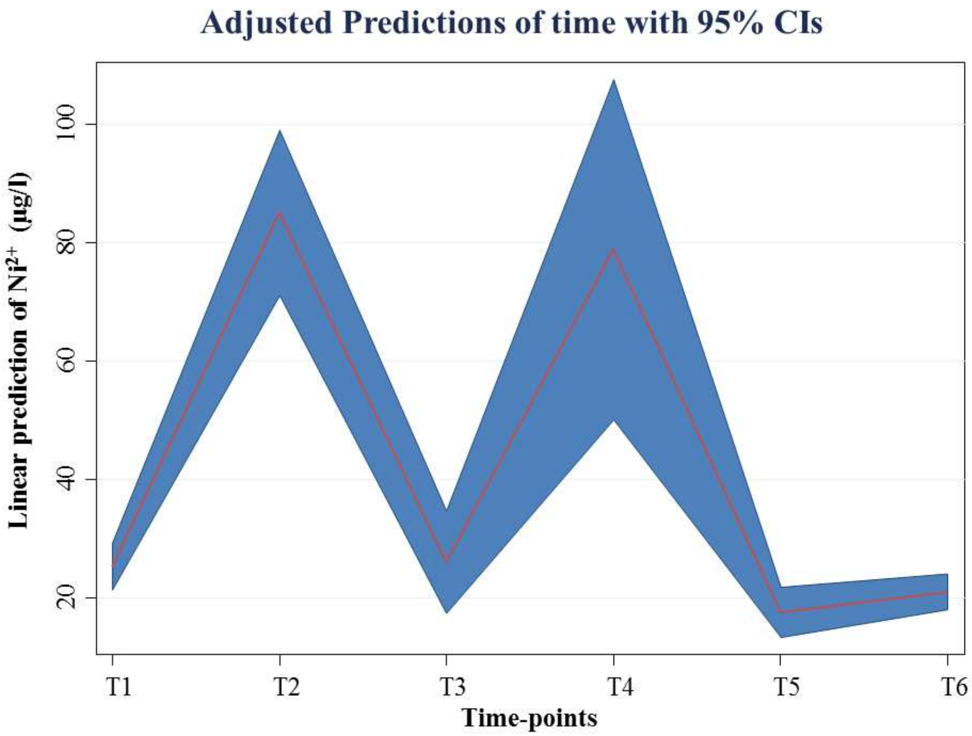
Fig. 4. Predicted marginal effects with associated 95% confidence intervals from the generalised estimating equations model for all patients according to time of sample collection: T1, reference standard; T2, after self-ligating bracket/band placement; T3, 2 weeks later and before wire insertion; T4, directly after wire insertion; T5, 4 weeks after wire insertion; T6, 8 weeks after wire insertion.

Fig. 4. Prognostizierte Randeffekte bei einem Konfidenzintervall von 95% mit dem angenommenen generalisierten Gleichstellungsmodell für alle Patienten in Abhängigkeit vom Zeitpunkt der Probenentnahme:

T1, Referenzstandard; T2, nach Insertion selbstligierender Brackets/Bänder; T3, 2 Wochen nach und direkt vor Bogeninsertion; T4, direkt nach Bogeninsertion; T5, 4 Wochen nach Bogeninsertion; T6, 8 Wochen nach Bogeninsertion.



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Supplementary Material

Supplement 1. Protocol for the placement of the fixed orthodontic appliances.

Teeth were polished with pumice and rubber cup to remove pellicle and food debris and afterwards were rinsed with water and dried with air. Preformed stainless steel bands (Ormco, Glendora, CA) were fitted to the patients' upper and lower first molars and cemented with glass-ionomer cement (Ketac Cem mu, 3M ESPE, Seefeld, Germany). The buccal surfaces of the rest of the teeth were treated with acid etched (phosphoric acid 37%, Ormco) for 15 seconds, rinsed again with water, and dried with air. Monomer primer (bonding) (Transbond XT, 3M Unitek, Monrovia, CA) was applied on etched surfaces of the teeth followed by application of light cure composite resin (Transbond XT, 3M Unitek, Monrovia, CA) on the bracket bases. Brackets (SmartClip™, 3M Unitek, Monrovia, CA) were pressed lightly on the tooth surface and material excess was removed. Polymerization was done with a halogen lamp for 30 seconds per tooth.